D E C L A R A T I O N

I, Ippei Andoh, 5-1-803, Takabashi, Koto-ku, Tokyo, Japan, do hereby declare that I am well acquainted with the Japanese language and English language and the attached English document is believed to be full, true, and faithful translation made by me of Japanese Patent Application 59183/1989. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Tokyo, Japan

This 29th day of November, 1991

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Ippei Andoh

PATENT OFFICE

JAPANESE GOVERNMENT

This is to certify that the annexed is a true copy of the following application as filed with this Office.

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March 23, 1990

Commissioner, Patent Office

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Application for Patent

March 10, 1989

(14,000Yen)

To: Commissioner,
Patent Office
Fumitake Yoshida

1. Title of the Invention

NOVEL PHYSIOLOGICALLY ACTIVE POLYPEPTIDE AND ITS USE

2. Number of Inventions described in Claims: 2

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6. List of Appended Documents:

(1) Specification	one copy
(2) Duplicate copy of the Petition	one copy
(3) Power of Attorney	each one copy
(4) Drawings	one copy

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Specification

1. Title of the invention

PHYSIOLOGICALLY ACTIVE POLYPEPTIDE AND USAGE OF THE SAME

2. What is claimed is:

1. A physiologically active polypeptide represented by formula (I) or a salt thereof,

X-Cys-Phe-Gly-Arg-Lys-Met-Asp-Arg-Ile-Ser-Ser-Ser-Ser-Gly-Leu-Gly-Cys-Lys-Val-Leu-Arg-Arg-His-OH (I)

wherein X is H, H-Gly-Ser-Gly-, or H-Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly-.

2. A pharmaceutical composition for curing circulation diseases which comprises the physiologically active polypeptide or a salt thereof defined in Claim 1.

3. Detail description of the invention

[Field of Industrial Application]

This invention relates to a physiologically active polypeptide and its use, and, more particularly, to a human brain natriuretic polypeptide and to a pharmaceutical composition for treating and curing circulation diseases such as hypertension, edemas, cardiac and renal failure comprising said polypeptide.

[Prior art]

Recently, a new peptide having diuretic activity was isolated in a purified form from porcine brain. Its

structure was determined and the peptide was named "porcine brain natriuretic peptide" (hereinafter referred to as porcine BNP or pBNP) [Sudo et al; *Nature*, *332*, 78-80 (1988)] since its structure and physiological activities are quite similar to those of artium natriuretic polypeptide (hereinafter referred to as ANP). BNP was revealed to have the control function on the body fluid volume, the balance of the electrolyte and blood pressure in the living body. This fact proved the existence of the double control mechanism by these ANP and BNP in the living body.

On the other hand, cloning of cDNA possessing a base sequence encoding porcine BNP has been realized and the structure of its precursor has been clarified [Maekawa et al; *Biochem. Biophys. Res. Commun. 157 (1)*, 410-416 (1988)].

Furthermore, cloning of cDNA encoding the human BNP was attempted employing the cDNA encoding the pBNP as a probe. The structural analysis of this cDNA led to the reasonable assumption of the amino acid sequence of the human BNP.

[Problems to be solved by the Invention]

Isolation or synthsis of human BNP, however, has never been reported. The effects or physiological activities of human BNP have been also unknown. Therefore, it is very significant to synthesize human BNP and investigate its physiological activities and find its usefulness by comparing with the known natriuretic peptide. Such study is important for the development of medicines in light of avoiding undesirable side effects such as antibody

production.

[Means to solve the Problems]

Based on the above-described assumption of the amino acid sequence in human BNP, the present inventors have synthesized various human BNPs and have studied their pharmacological activities. As a result, the inventors have found that these BNPs had excellent smooth muscle relaxation and natriuretic activities, and finally completed the present invention.

Accordingly, an object of this invention is to provide a physiologically active polypeptide represented by formula (I) or a salt thereof,

X-Cys-Phe-Gly-Arg-Lys-Met-Asp-Arg-Ile-Ser-Ser-Ser-Ser-Gly-
Leu-Gly-Cys-Lys-Val-Leu-Arg-Arg-His-OH (I)

wherein X is H, H-Gly-Ser-Gly-, or H-Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly-, and a pharmaceutical composition for curing circulation diseases which comprises such a polypeptide or a salt thereof.

In this specification, the peptide of formula (I) having H-Gly-Ser-Gly- for X may be referred to as human BNP-26 and that having H-Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly- for X may be referred to as human BNP-32.

The abbreviated names of the peptide constituents in this specification are being commonly used in the art and

have the following meaning.

Asp : L-Aspartic acid

Ser : L-Serine

Gly : Glycine

Cys : L-Cystein

Phe : L-Phenylalanine

Arg : L-Arginine

Leu : L-Leucine

Ile : L-isoluecine

Asn : L-Asparagine

Val : L-Valine

Tyr : L-Tyrosine

The peptide of the present invention represented by formula (I) can be prepared by the solid phase method or the liquid phase method which are conventionally used in the art [e.g. N. Izumiya, et al. "*Peptide Synthesis*", Maruzen Publishing Co., Ltd. (1984); "*Lecture of Biochemistry Experiment (I), Protein Chemistry*" edited by Chemical Society of Japan, vol. 4, 208-495 (1977), published by Tokyo Kagaku Dojin].

When the peptide (I) is prepared by the solid phase method, the following protective groups of amino acid can preferably be used; i.e., 9-fluorenylmethyloxycarbonyl (Fmoc) group for the α-amino group, tert-butyl (tBu) group for the β-carboxyl group of aspartic acid, 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) group for the guanidino group of arginine, tert-butyl (tBu) group for the hydroxyl group

of cysyeine, trityl group (Trt) for the imidazole group of histidine, and tert-butyloxycarbonyl (Boc) group for the ε-amino group of lysine. *p*-Alkoxybenzyl alcohol resin (Wang Resin) is a preferable insoluble resin for use. Preferable methods used for the condensation of protected amino acids are the dicyclohexylcarbodiimide (DCC) method, the active ester method using 1,3-diisopropylcarbodiimide (DIC), the acid anhydride method using DCC, the diphenylphosphoryl azide (DPPA) method, and the like. The protective groups are not limited to those given above, and α-amino group of amino acids, for example, may be protected by tert-butyloxycarbonyl (Boc) group.

Production of the polypeptide of the present invention by the solid phase method can be carried out, for example, by the following manner. The protected derivative Fmoc-His(Trt)-OH in which His is the C-terminal amino acid of the polypeptide is first introduced into *p*-alkoxybenzyl alcohol resin. The corresponding protected amino acids are successively combined in this way to synthesize a protected peptide resin. Subsequently, removal of peptide from the resin and elimination of protective groups other than Acm are concurrently performed by the treatment with piperidine and trifluoroacetic acid (TFA), the treatment with piperidine and trimethylsilyl bromide (TMSBr) [*Chem. Pharm. Bull.*, 35, (9), 3880 (1987)], and the like to obtain a peptide having an Acm group for thiol of cystein, such a peptide being referred to as Cys(Acm)-peptide. Then, the

Cys(Acm)-peptide is oxidized with iodine to remove the thiol protective group and, at the same time, to form a disulfide bond between two thiol groups of cystein in the peptide molecule, thus producing crude polypeptide of the present invention.

The crude polypeptide is purified by a conventional manner such as, for example, gel filtration, ion exchange chromatography, reversed phase HPLC, or the like.

The peptide of formula (I) of the present invention can be converted into an acid addition salt according to a conventional manner using an inorganic acid such as hydrochloric acid, sulfuric acid, phosphoric acid, or the like; or an organic acid such as formic acid, acetic acid, citric acid, tartaric acid, fumaric acid, maleic acid, or the like.

[Action and Effect of the Invention]

The peptide of formula (I) of the present invention thus produced possesses smooth muscle relaxation and other activities. These activities were investigated as follows:

<Smooth muscle relaxation activity>

(1) Test method

Rectum of a chicken, age 4-7 days, was enucleated and muscle specimens, 1.5 cm long, were prepared. The specimens were immersed into 2.5 ml of Krebs-Henseleit solution, containing 2×10^{8} M carbachol, which was aerated with a 95% O_2-5% CO_2 mixed gas and maintained at 32°C in a 3 ml organ bath. The specimens were allowed to equilibrate under a 0.5

g static tension for about 30 minutes. When the self-acting of the muscle became constant, 100 ng of human BNP-26 was added and relaxation of the muscle was measured for 6-8 minutes after the addition. After the measurement, the specimens were rinsed two or three times, and, after 20-30 minutes, the above procedure was repeated using 200 ng of human BNP-32. The human BNPs were used dissolved in a prescribed amount of physiological saline.

(2) Results

The results are shown in Figures 1-A and 1-B. From the results, the polypeptide of the present invention was found to exhibit strong smooth muscle relaxation activity at a dose of 100-200 ng.

As mentioned above, the peptide or the salt thereof produced by the present invention possesses excellent smooth muscle relaxation activity, diuretic or natriuretic activity, and vasodepressor activity. The BNP is safe as a medicine for humans because it is derived from human, thus it can be used as a medicine for curing such diseases as cardiac edema, nephric edema, hepatic edema, pulmonary edema, hypertension, congestive heat failure, acute and chronic renal failure, and the like.

Any methods conventionally used for the administration of peptide medicines, e.g. intravenous injection, intramuscular injection, subcutaneous injection, sublingual administration, intracutaneous administration, rectum administration, or the like, can be employed for the

administration of the peptide of the present invention.

A preferable dose, that can avoid dangerous or harmful side effects, is 0.5 μg/kg to 100 mg/kg.

[Examples]

The exemplary embodiments are given for illustration of the invention and are not intended to be limiting thereof.

Example 1

(1) Synthesis of peptide human BNP-26 and human BNP-32

(a) Synthesis of a protected peptide resin

For the synthesis of the protected peptide resin all α-amino groups of amino acids were protected by 9-fluorenylmethyloxycarbonyl (Fmoc) group, and among active side chains, the β-carboxyl group of aspartic acid was protected by tert-butyl (tBu) group, the guanidino group of arginine was protected by 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) group, the hydroxyl group of serine was protected by tert-butyl (tBu) group, the thiol group of cysteine was protected by acetamidomethyl (Acm) group, the imidazole group of histidine was by trityl group (Trt), and the ε-amino group of lysine was by tert-butyloxycarbonyl (Boc) group. 1.0 g of *p*-alkoxybenzyl alcohol resin into which protective His group was introduced was used as the resin.

In the condensation of the protected amino acid, the Fmoc group which is the protected group for the N-terminal amino acid of the protected peptide bonding to the resin was

almost completely removed by the treatment with piperidine, repeated twice, at room temperature for 6 minutes. The free amino group from which the Fmoc group was eliminated was condensed with the carboxyl group of the Fmoc protected amino acid located next in the sequence of the target peptide. The condensation of the protected amino acid was carried out by treating 1 mmol of Fmoc-protected amino acid with 1,3-diisopropylcarbodiimide (DIC) in the presence of 1-hydroxybenztriazole. The same procedure was repeated when the reaction was not completed by this treatment. The progress and completion of the reaction were monitored by the Keizer test using ninhydrin.

Fmoc-Gly-Ser(tBu)-Gly-Cys(Acm)-Phe-Gly-Arg(Mtr)-Lys(Boc)-Met-Asp(tBu)-Arg(Mtr)-Ile-Ser(tBu)-Ser(tBu)-Ser(tBu)-Ser(tBu)-Gly-Leu-Gly-Cys(Acm)-Lys(Boc)-Val-Leu-Arg(Mtr)-Arg(Mtr)-His(Trt)-resin was thus synthesized. At this stage, a portion of the product was taken out and the Fmoc group was removed in the same manner as described above, thus obtaining 670 mg of H-Gly-Ser(tBu)-Gly-Cys(Acm)-Phe-Gly-Arg(Mtr)-Lys(Boc)-Met-Asp(tBu)-Arg(Mtr)-Ile-Ser(tBu)-Ser(tBu)-Ser(tBu)-Ser(tBu)-Gly-Leu-Gly-Cys(Acm)-Lys(Boc)-Val-Leu-Arg(Mtr)-Arg(Mtr)-His(Trt)-resin (hereinafter referred to as "protected human BNP-26 resin").

The remaining resin was further subjected to N-terminal extension reaction to obtain Fmoc-Ser(tBu)-Pro-Lys(Boc)-Met-Val-Gln-Gly-Ser(tBu)-Gly-Cys(Acm)-Phe-Gly-Arg(Mtr)-Lys(Boc)-Met-Asp(tBu)-Arg(Mtr)-Ile-Ser(tBu)-Ser(tBu)-

Ser(tBu)-Ser(tBu)-Gly-Leu-Gly-Cys(Acm)-Lys(Boc)-Val-Leu-Arg(Mtr)-Arg(Mtr)-His(Trt)-resin. The Fmoc group was removed in the same manner as described above, thus obtaining 1.5 g of H-Ser(tBu)-Pro-Lys(Boc)-Met-Val-Gln-Gly-Ser(tBu)-Gly-Cys(Acm)-Phe-Gly-Arg(Mtr)-Lys(Boc)-Met-Asp(tBu)-Arg(Mtr)-Ile-Ser(tBu)-Ser(tBu)-Ser(tBu)-Ser(tBu)-Gly-Leu-Gly-Cys(Acm)-Lys(Boc)-Val-Leu-Arg(Mtr)-Arg(Mtr)-His(Trt)-resin (hereinafter referred to as "protected human BNP-32 resin").

(b) Synthesis of Cys(Acm)-human BNP-26

The protected human BNP-26 resin (600 mg) and 2.4 ml of thioanisole were placed in a reactor, and to this 20 ml of trifloroacetic acid (TFA), 2.6 ml of trimethylsilyl bromide (TMSBr), and 2.4 ml of ethanedithiol were added and reacted at 0°C for 3 hours. After the reaction, the resultant reaction mixture was washed with 200 ml of ether to remove anisole, and the product was extracted with 20 ml of 1 N acetic acid. The resin and the insoluble substance was removed by centrifugation. To the residue was added 1 ml of 1 M sodium fluoride (NaF) with cooling. The mixture was adjusted to pH 8 with 5% aqueous ammonia using a Universal test paper and left for 30 minutes. After adjusting to pH 5 with 1 N acetic acid, the mixture was diluted with water to a volume of 10-fold, absorbed to a column (ϕ 3 cm x 8.5 cm) packed with 60 ml of ODS resin (LC-Sorb: trademark, product of Chemco Co.), washed thoroughly with 0.1 N acetic acid, and eluted with 200 ml of 60% acetonitrile containing 0.1%

TFA. The acetonitrile was evaporated under reduced pressure and the residue was freeze-dried to obtain 300 mg of crude Cys(Acm)-human BNP-26.

The crude product was dissolved into 9 ml of 1 N acetic acid and the solution was subjected to reversed phase HPLC over a nucleosil 120-5C18 column (20 x 250 mm) in 9 portions at a flow rate of 5 ml/min. Solvent (A), a mixture of water:acetonitrile:10% TFA = 90:10:1, and Solvent (B) a mixture of water:acetonitrile:10% TFA = 40:60:1, were used at a linear gradient from (A):(B)=90:10 to (A):(B)=55:45 for 120 minutes. This procedure was repeated 9 times and the main peak eluted at 57-61 minute was collected. Acetonitrile was evaporated from the collected fraction and the residue was freeze-dried to obtain 96.0 mg of Cys(Acm)-human BNP-26.

(c) Synthesis of human BNP-26

Solution A was prepared by dissolving 227 mg of iodine into 50 ml of 95% acetic acid and by adding 80 µl of 1 N hydrochloric acid.

Solution B was prepared by dissolving 2.1 g of citric acid and 575 mg of L-ascorbic acid into 10 ml of 2 N sodium hydroxide, and made up to the final volume of 50 ml by an addition of water.

A solution 89.0 mg of Cys(Acm)-human BNP-26 in 5 ml of 90% acetic acid was added dropwise into 30 ml of Solution A at room temperature while stirring. After the addition, the mixture was stirred for a further 20 minutes. To this

mixture Solution B was added dropwise until the brown color of iodine disappeared. The resulting solution was diluted with 500 ml of water and applied to a column (ϕ 2 cm x 9.5 cm) packed with 30 ml of ODS resin (LC-Sorb: trademark, product of Chemco Co.). The column was washed thoroughly with 0.1 N acetic acid, and eluted with 60 ml of 60% acetonitrile containing 0.1% TFA. The acetonitrile was evaporated under reduced pressure and the residue was freeze-dried to obtain 60.0 mg of crude human BNP-26.

The crude product was dissolved into 4 ml of 1 N acetic acid and the solution was applied to reversed phase HPLC over a nucleosil 120-5C18 column (20 x 250 mm) in 4 divided portions at a flow rate of 5 ml/min. Linear gradient elution was carried out using Solvent (A), a mixture of water:acetonitrile:10% TFA = 90:10:1, and Solvent (B), a mixture of water:acetonitrile:10% TFA = 40:60:1, from (A):(B)=90:10 to (A):(B)=55:45 for 120 minutes. This procedure was repeated 4 times and the main peak eluted at 62-66 minute was collected. Acetonitrile was evaporated from the collected fraction and the residue was freeze-dried to obtain 25 mg of human BNP-26.

(d) Synthesis of Cys(Acm) human BNP-32

Release from the resin and removal of the protected group was performed in the same manner as described in (b) on 700 mg protected human BNP-32 using thioanisole, TFA, TMSBr, and ethanedithiol. The product was purified over reversed phase HPLC to obtain 60.0 mg of Cys(Acm)-human

BNP-32.

(e) Synthesis of human BNP-32

60.0 mg of Cys(Acm)-human BNP-32 was subjected to the Acm removal and cyclization in the same manner as (c) using iodine to obtain 20.0 mg of crude human BNP-32. The crude product was dissolved into 4 ml of 1 N acetic acid and the solution was subjected to reversed phase HPLC over a nucleosil 120-5C18 column (20 x 250 mm) in 4 portions at a flow rate of 5 ml/min. Elution was carried out linear gradiently using Solvent (A), a mixture of water:acetonitrile:10% TFA = 90:10:1, and Solvent (B) a mixture of water:acetonitrile:10% TFA = 40:60:1, from (A):(B)=90:10 to (A):(B)=55:45 for 120 minutes. This procedure was repeated 4 times and the main peak eluted at 61-64 minute was collected. Acetonitrile was evaporated from the collected fraction and the residue was freeze-dried to obtain 5 mg of human BNP-32.

(2) Physicochemical Characteristics

Physicochemical characteristics of human BNP-26 and human BNP-32 prepared in (1) above were as follows:

(a) Form

White powder

(b) Solubility in solvents

Soluble in water, acidic aqueous solutions, and acetic acid. Insoluble in chloroform, benzene, ethyl ether, and hexane.

(c) Property

basic

(d) Amino acid composition

Given in Table 1.

TABLE 1

Peptide	Human BNP-26	Human BNP-32
Molecular Weight	2793.28	3464.12
Amino Acid Composition *	Measured (Calculated)	Measured (Calculated)
Asp + Asn	1.06 (1)	0.94 (1)
Ser	4.61 (5)	4.55 (6)
Glu + Gln	-	0.94 (1)
Gly	5.33 (5)	4.56 (5)
Cys **	1.62 (2)	1.61 (2)
Val	0.90 (1)	1.81 (2)
Met	1.02 (1)	1.81 (2)
Ile	0.94 (1)	0.93 (1)
Leu	1.98 (2)	1.87 (2)
Phe	1.00 (1)	1.00 (1)
Lys	1.97 (2)	2.71 (3)
His	1.00 (1)	0.95 (1)
Arg	3.99 (4)	3.78 (4)
Pro ***	-	1.08 (1)

* The measured value (mols) in the table is one example of amino acid analysis.

** Measured as Cys-SO_3H after the oxidation with performic acid, followed by hydrolysis. Other amino acids were hydrolyzed with 6 N HCl.

*** Measured at 440 nm.

4. Brief Description of the Drawings

Figure 1-A shows the change in relaxation length with time of a chicken rectum specimen to which 100 ng of human BNP-26 of the present invention was administered.

Figure 1-B shows the change in relaxation length with time of a chicken rectum specimen to which 200 ng of human BNP-26 of the present invention was administered.

... Concluded ...

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FIG. 1-A

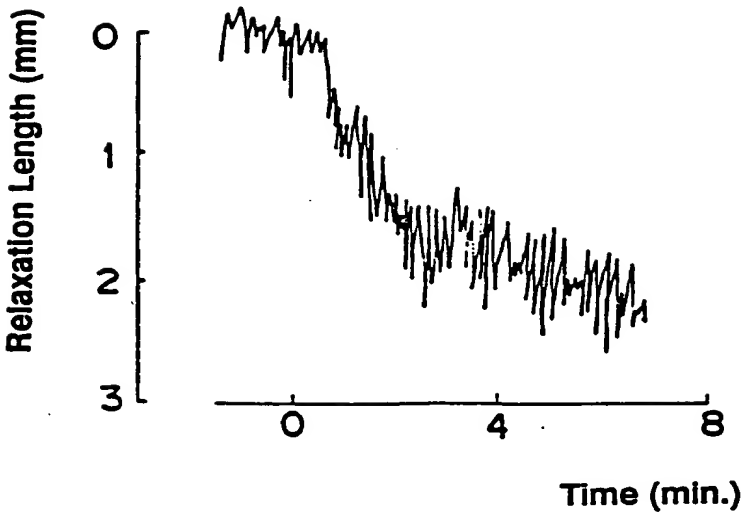
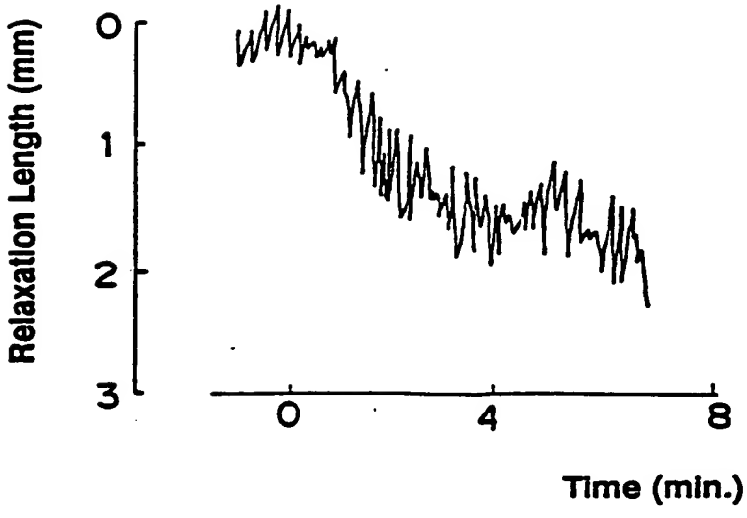

Relaxation Length (mm)
0
1
2
3
0
4
8
Time (min.)

FIG. 1-B


Relaxation Length (mm)
0
1
2
3
0
4
8
Time (min.)